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Construction of new *Campylobacter* cloning vectors and a new mutational *cat* cassette

(Site-specific mutagenesis; recombinant DNA; multiple cloning site; PCR; shuttle vectors)

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SUMMARY

We have developed new *Campylobacter* shuttle vectors which are 6.5–6.8-kb plasmids carrying *Campylobacter* and *Escherichia coli* replicons, a multiple cloning site (MCS), the *lacZα* gene, *oriT* and either a kanamycin or chloramphenicol resistance-encoding gene (Km^R or Cm^R) from *Campylobacter* which functions in both hosts. These vectors can be mobilized efficiently from *E. coli* into *C. jejuni* or *C. coli*, and stably maintained in these hosts. Plasmids pRY107 and pRY108 carry a Km^R marker and 17 unique cloning sites in two different orientations in *lacZα*, allowing easy blue/white color selection. Plasmids pRY111 and pRY112 contain a Cm^R gene and 17 unique sites in both orientations. In addition, MCS are flanked by T7 and T3 late promoters and M13 forward and reverse primer sites, facilitating expression in T7 or T3 expression systems and sequence analysis. A *Campylobacter* Cm^R gene cartridge, bracketed by six restriction sites, has been developed for use in site-specific mutagenesis of *Campylobacter* genes.

INTRODUCTION

Systems of experimental genetics are in the early stages of development for the important gastrointestinal pathogens *Campylobacter jejuni* and *C. coli*. Standard genetic techniques developed for the *Enterobacteriaceae* have, for the most part, not been successfully adapted for campylobacters. *E. coli* plasmids, even from broad-host-range compatibility groups, fail to replicate in *Campylobacter* spp., although P incompatibility plasmids can mobilize shuttle vectors containing *E. coli* and *C. coli* plasmid replicons and an origin of transfer (*oriT*) from *E. coli* into *Campylobacter* spp. (Labigne-Roussel et al., 1987). To date, only two shuttle vector series are available for cloning of campylobacter genes. Plasmid pILL550 (Labigne-Roussel et al., 1987) (8.5 kb), has a MCS with four restriction sites, although none of these sites allows for insertional inactivation of a marker. The pUOA series of shuttle vectors (Wang and Taylor, 1990a,b), derived from pILL550, contain the polylinker and *lacZα* gene from pUC13, and either Km^R, Tc^R or Cm^R markers and range in size from 7.4 kb to 11.8 kb. However, due to sites within the campylobacter portion of these vectors, only three restriction sites in the *lacZ* MCS remain unique. The aim of this study was to construct a series of new shuttle vectors which carry alternate antibiotic markers (Km or Cm) and an improved MCS that would facilitate cloning of campylobacter genes in *E. coli*, and their return to *Campylobacter* spp. for functional analyses. In addition,

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Abbreviations: Ap, ampicillin; bp, base pair(s); *C.*, *Campylobacter*; CAT, Cm acetyltransferase; *cat*, gene encoding CAT; Cm, chloramphenicol; kb, kilobase(s) or 1000 bp; Km, kanamycin; LB, Luria-Bertani medium (agar); MCS, multiple cloning site(s); Mob^+, capable of being mobilized conjugatively; nt, nucleotide(s); *ori*, origin of DNA replication; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; ^{R}, resistance; ss, single strand(ed); Tc, tetracycline; Tm, trimethoprim; [ ], denotes plasmid-carrier state.

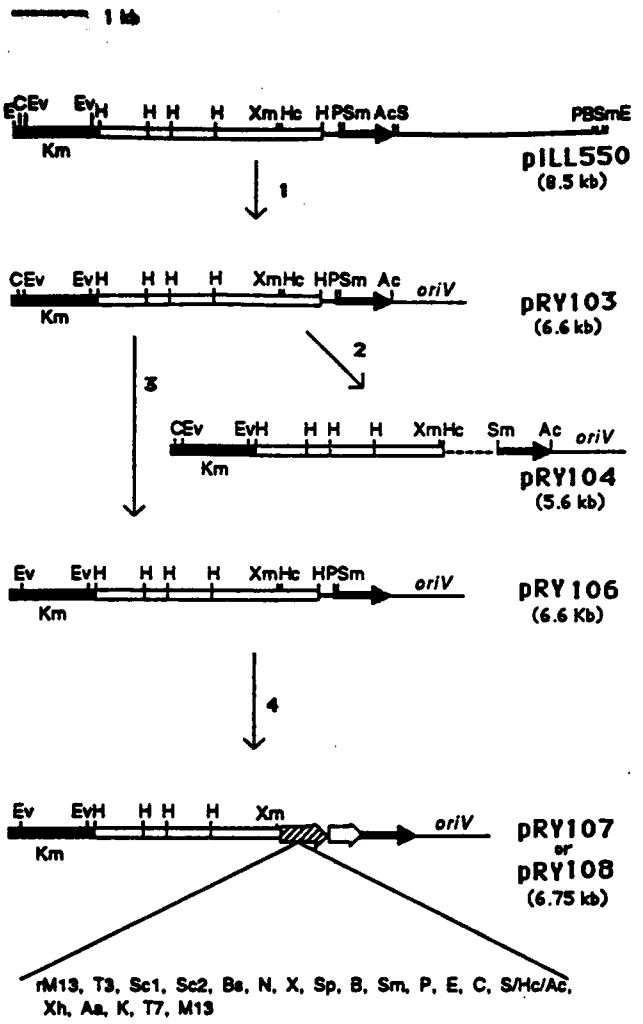

Fig. 1. Construction of shuttle vectors pRY107 and pRY108. Step 1: pILL550 [which consists of 3.1-kb of pBR322, the 3.24-kb cryptic campylobacter plasmid pIP1455 (Lambert et al., 1985), the 1.4-kb campylobacter Km^R gene, and the 760-bp *oriT* (Labigne-Roussel et al., 1987)] was digested with *Eco*RI + *Sal*I to excise a 5.5-kb fragment containing the Km^R gene, *oriT* and all of pIP1455. This fragment was blunted with PolIk, and ligated to a 1.1-kb *Ava*I-*Pst*I fragment (also blunted with PolIk) containing *oriV* of pBR328 to generate pRY103. Step 2: pRY103 was digested with *Hin*cII + *Sma*I and self-ligated generating pRY104, which was demonstrated to retain the ability to replicate in *Campylobacter* spp. Step 3: *Acc*I and *Cla*I sites of pRY103 were sequentially eliminated resulting in pRY106. Step 4: pRY106 was digested with *Hin*cII + *Sma*I and ligated to a 1.15-kb fragment from pWSK29 containing the MCS and F1 *ori* to generate pRY107 or the corresponding fragment from pWKS30 to generate pRY108. Plasmid transformants were selected in *E. coli* DH5α on LB agar containing 100 μg Km/ml. Constructions were transformed into DH5α[RK212.2] (Figurski and Helinski, 1979) and mobilized into *C. coli* VC167 as previously described, selecting on Mueller-Hinton agar supplemented with 10 μg Tm/ml and 100 μg Km /ml (Guerry et al., 1991). Symbols: (—), deleted sequence; Km, campylobacter Km^R gene; open box, pIP1455 DNA; solid arrow, *oriT*; hatched arrow, MCS and *lacZ* gene; open arrow, F1 *ori*. Aa, *Apa*I; Ac, *Acc*I; B, *Bam*HI; Bs, *Bst*XI; C, *Cla*I; E, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; Hc, *Hin*cII; K, *Kpn*I; N, *Not*I; P, *Pst*I; S, *Sal*I; Sc1, *Sac*I; Sc2, *Sac*II; Sm, *Sma*I; Sp, *Spe*I; X, *Xba*I; Xh, *Xho*I; Xm, *Xmn*I. Other sites are abbreviated as follows: rM13, reverse M13 priming site; T3, T3 promoter; T7, T7 promoter; M13, forward M13 priming site. Only the remaining unique restriction sites are shown below pRY107; those in pRY108 are in reverse order.

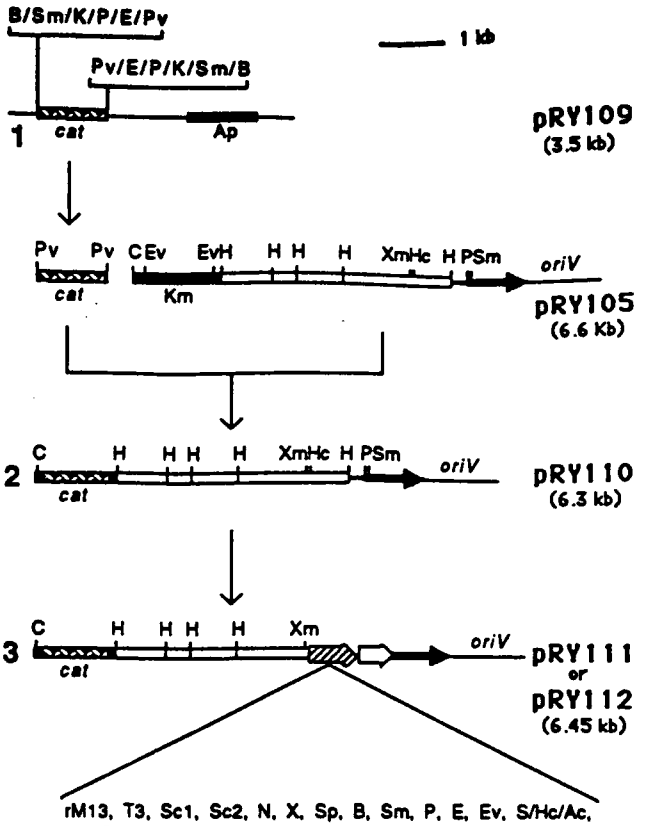

Fig. 2. Construction of a *cat* cassette with MCS and shuttle vectors pRY111 and pRY112. (1) Two 58-mer primers, RAA17 (5'-ATTATTAGGATCCCGGGTACCTGCAGAATTCAGCTGCTCGGCGGTGTTCCTTTCCAAG) and RAA18 (5'-ATTATTAGGATCCCGGGTACCTGCAGAATTCAGCTGCGCCCTTTAGTTCCTAAAGGGT) were synthesized on an Applied Biosystems Model 392 DNA/RNA synthesizer. These two primer sequences were modified from the known nt sequence of the campylobacter *cat* gene (Wang and Taylor, 1990a). The RAA17 primer is located 70 bp upstream from the ATG start codon of the *cat* gene and the RAA18 primer at 26 bp downstream from the stop codon. At the 5' ends, both primers have added sequences encoding restriction sites for *Bam*HI/*Sma*I/*Kpn*I/*Pst*I/*Eco*RI/*Pvu*II. A PCR reaction was carried out in a total volume of 100 μl containing 200 ng of pUOA18 DNA (a clone of the campylobacter *cat* gene; Wang and Taylor, 1990a)/10 μl of 10 × buffer/2 μl of 10 mM of each dNTP/200 ng of each primer/2.5 units of *Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT). The samples were overlaid with paraffin oil, and subjected to 30 cycles of amplification in a programmable thermocycler. The conditions of amplification were: 94°C for 1 min, 45°C for 2 min, and 72°C for 1 min. The predicted 0.8-kb DNA PCR product was recovered from a 0.7% agarose gel, digested with *Bam*HI, and cloned into the *Bam*HI site of pUC18 to generate plasmid pRY109. (2) pRY105 (Table I) was digested with *Eco*RV to delete the Km^R gene and ligated to the *cat* gene (0.8 kb) excised from plasmid pRY109 (Table I) as a *Pvu*II fragment to generate pRY110. (3) pRY110 was digested with *Hin*cII + *Sma*I and ligated to the MCS of pWSK29 to generate pRY111 and to the MCS of pWKS30 to generate pRY112. Plasmid transformants were selected in *E. coli* DH5α on LB agar containing 20 μg Cm/ml. Constructions were transformed into DH5α[RK212.2] (Figurski and Helinski, 1979) and mobilized into *C. coli* VC167 as previously described selecting on Mueller-Hinton agar supplemented with 10 μg Tm/ml and 20 μg Cm/ml (Guerry et al., 1991). D, *Dra*II; Pv, *Pvu*II. For other designations, see Fig. 1. Only the unique remaining restriction sites are shown below pRY111; those in pRY112 are in reverse order.

TABLE I
Characterization of plasmids constructed for this study

Plasmids	Size (kb)	Phenotype/Characteristics	Source	Reference
pRY103	6.6	Km^R Mob^- (Fig. 1)	pILL550 pBR328	Labigne-Roussel et al. (1987) (Fig. 1) Soberon et al. (1980)
pRY104	5.6	Km^R, deletion of 1-kb *HincII-SmaI* fragment, Mob^- (Fig. 1)	pRY103	This work
pRY105	6.6	Km^R, *AccI* site deleted (Fig. 2)	pRY103	This work
pRY106	6.6	Km^R, *AccI* and *ClaI* sites deleted (Fig. 1)	pRY105	This work
pRY107	6.75	Km^R, pWSK29 MCS, 17 unique sites (Fig. 1)	pRY106	This work
pRY108	6.75	Km^R, pWKS30 MCS, 17 unique sites (Fig. 1)	pRY106	This work
pRY109	3.5	Ap^R, Cm^R, *cat* gene in *BamHI* site of pUC18	Fig. 2	This work
pRY110	6.3	Cm^R, Mob^- (Fig. 2)	pRY105	This work
pRY111	6.45	Cm^R, pWSK29 MCS, 17 unique sites (Fig. 2)	pRY110	This work
pRY112	6.45	Cm^R, pWKS30 MCS, 17 unique sites (Fig. 2)	pRY110	This work

we have constructed a new cassette for insertional mutagenesis in *Campylobacter* spp. which consists of the *C. jejuni cat* gene (Wang and Taylor, 1990a) bracketed by polylinkers (MCS).

EXPERIMENTAL AND DISCUSSION

(a) Construction of pILL550 derivatives

Parts of the original 8.5-kb campylobacter cloning vector, pILL550, were deleted as described in Fig. 1 to generate plasmid pRY103. Plasmid pRY103 was mobilized from *E. coli* donor cells containing the P incompatibility conjugative plasmid RK212.2 (Figurski and Helinski, 1979) into *C. coli* VC167 and *C. jejuni* 81176 by a modification of the conjugation method of Labigne-Roussel et al. (1987). Plasmid DNA was extracted from selected transconjugants to confirm the presence of pRY103 extrachromosomally in the campylobacter recipients. Restriction analyses of pRY103 indicated the presence of additional *XmnI*, *HincII*, *PstI*, *SmaI* and *AccI* sites in the pILL550 portion of the molecule which have not been reported previously (Fig. 1).

(b) Construction of pRY107 and pRY108 cloning vectors and derivatives

Labigne-Roussel et al. were unable to further reduce the size of the *C. coli* plasmid pIP1455 component of pILL550 (see Fig. 1), suggesting that discrete loci were required for replication in campylobacters (Labigne-Roussel et al., 1987). In an attempt to reduce the size of the cloning vector, a 1.0-kb *HincII-SmaI* fragment of pRY103 (Fig. 1) which includes part of pIP1455 and part of pBR322, was deleted generating pRY104 (Fig. 1, Table I). Plasmid pRY104 could still be mobilized into and replicate in *C. coli* VC167, indicating that deletion of this 1.0-kb fragment did not affect replication in *Campylobacter*.

The *AccI* and *ClaI* sites in plasmid pRY103 were sequentially eliminated by restriction enzyme digestion, blunt ending with PolIk, and self-ligation to generate pRY105 and finally pRY106 (Table I). MCS and an F1 phage *ori* from plasmids pWSK29 or pWKS30 (Wang and Kushner, 1991; originally taken from pBluescript, Stratragene, La Jolla, CA, USA) were then inserted between the *HincII* and *SmaI* sites of pRY106. The resulting plasmids, which contain 17 unique sites in *lacZ*α gene in two orientations, were named pRY107 and pRY108 (Fig. 1, Table I). Their ability to be mobilized from *E. coli* and replicate within *Campylobacter* spp. was also confirmed.

(c) Construction of *cat* gene cassette by PCR

A *cat* cassette bracketed by polylinkers was constructed and cloned into pUC18 to generate pRY109 as described in Fig. 2. The clone conferred Cm^R to the *E. coli* host, and the presence of all of the expected restriction sites bracketing the *cat* gene was confirmed.

(d) Construction of new *cat* shuttle vectors

The Km^R gene of pRY105 (Table I) was replaced with the *cat* gene in the following manner. Plasmid pRY105 was digested with *Eco*RV to delete 1.1 kb of Km^R. The 5.5-kb *Eco*RV vector fragment was ligated to the 0.8-kb *cat* gene excised from pRY109 by digestion with *PvuII*. The resulting plasmid was named pRY110 (Fig. 2, Table I). The 1.15-kb *SspI-BglII* fragments containing MCS from pWSK29 or pWKS30 were subsequently blunted with PolIk and inserted between the *HincII* and *SmaI* sites of plasmid pRY110 to generate the final constructs pRY111 and pRY112, respectively (Fig. 2 and Table I). Plasmids pRY111 and pRY112 (6.45 kb) confer Cm resis-

tance to both *E. coli* and *Campylobacter* hosts, and contain 17 unique restriction sites for cloning in two orientations in the *lacZ*α gene.

(e) Conclusions

(*1*) The campylobacter shuttle vectors described above are composites of pILL550 (Labigne-Roussel et al., 1987) and the MCS of the *E. coli* vectors of Wang and Kushner (1991). These vectors offer greater versatility and flexibility for cloning in *Campylobacter* spp. due to increased numbers of unique restriction sites and the ease of rapid color screening with the *lacZ*α gene. Since the polylinkers are flanked by T7 and T3 RNA polymerase promoters, M13 forward and reverse primer sites, the plasmids can be used for DNA sequencing with commercial primers. In addition, the T7 and T3 promoters could be used for generating RNA probes in vitro or for high level expression. The f1 *ori* can be used for production of ss DNA in the presence of a helper phage for rapid DNA sequencing and for making ss DNA probes. The campylobacter replicon has also been reduced in size by an additional 0.6 kb from that found in pILL550. The ultimate test of any vector depends on its record of actual usefulness. Some of these vectors have already been used in our laboratories for direct cloning of *C. jejuni* genes in *E. coli* and transfer into other strains of *Campylobacter* spp. (R.Y. and P.G., in preparation).

(*2*) The ability to perform site-specific mutations on campylobacter genes cloned in *E. coli* has been a major advance in campylobacter research (Labigne-Roussel et al., 1988; Guerry et al., 1990; 1991; Wassenaur et al., 1991). To date, the only cassette available for such mutational studies is the original Km^R cassette developed by Labigne-Roussel et al. The *cat* cassette described here offers additional flexibility and would allow for mutation of two genes in the same strain. Moreover, the existence of alternately marked vectors with a wide selection of unique restriction sites affords the possiblity of performing complementation analysis. Thus, if the *cat* cassette is used to mutate a chromosomal gene, one could utilize Km^R shuttle vectors for complementation analysis. The *cat* cassette has been used successfully in our laboratories to mutate several *C. jejuni* genes (R.Y. and P.G., in preparation).

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REFERENCES

Alm, R.A., Guerry, P., Power, M.E., Lior, H. and Trust, T.J.: Analysis of the role of flagella in the heat-labile Lior serotyping scheme of thermophilic campylobacters by mutant allele exchange. J. Clin. Microbiol. 29 (1991) 2438–2445.

Butzler, J.P. and Skirrow, M.B.: *Campylobacter* enteritis. Clin. Gastroenterol. 36 (1979) 737–765.

Chowdhury, M.N.H.: *Campylobacter jejuni* enteritis: a review. Trop. Geogr. Med. 36 (1984) 215–222.

Figurski, D.H. and Helinski, D.R.: Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in trans. Proc. Natl. Acad. Sci. USA 76 (1979) 1648–1652.

Georges-Courbot, M.C., Beraud-Cassel, A.M., Gouandjika, I. and Georges, A.J.: Prospective study of enteric *Campylobacter* infections in children from birth to 6 months in the Central African Republic. J. Clin. Microbiol. 25 (1987) 836–839.

Guerrant, R.L., Kirchhoff, L.V., Shields, D.S., Nations, K., Leslie, J., de Sousa, M.A., Araujo, J.G., Correia, L.L., Sauer, K.T., McClelland, K.E., Trowbridge, F.L. and Hughes, J.M.: Prospective study of diarrheal illnesses in northeastern Brazil: patterns of disease, nutritional impact, etiologies and risk factors. J. Infect. Dis. 148 (1983) 986–997.

Guerry, P., Logan, S.M. Thornton, S.A. and Trust, T.J.: Genomic organization and expression of *Campylobacter* flagellin genes. J. Bacteriol. 172 (1990) 1853–1860.

Guerry, P., Alm, R.A., Power, M.E. Logan, S.M., and Trust, T.J.: Role of two flagellin genes in *Campylobacter* motility. J. Bacteriol. 173 (1991) 4757–4764.

Labigne-Roussel, A., Courcoux, P. and Tompkins, L.: Gene disruption and replacement as a feasible approach for mutagenesis of *Campylobacter jejuni*. J. Bacteriol. 170 (1988) 1704–1708.

Labigne-Roussel, A., Harel, J. and Tompkins, L.: Gene transfer from *Escherichia coli* to *Campylobacter* species: development of shuttle vectors for genetic analysis of *Campylobacter jejuni*. J. Bacteriol. 169 (1987) 5320–5323

Lambert, T., Gerbaud, G., Trieu-Cuot, P. and Courvalin, P.: Structural relationship between the genes encoding 3'aminoglycoside phosphotransferases in *Campylobacter* and in Gram positive cocci. Ann. Inst. Pasteur Microbiol. 136B (1985) 134–150.

Soberon, X., Covarrubias, L. and Bolivar, F.: Construction and characterization of new cloning vehicles, IV. Deletion derivatives of pBR322 and pBR325. Gene 9 (1980) 287–305.

Wang, R.F. and Kushner, S.R.: Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene 100 (1991) 195–199.

Wang, Y. and Taylor, D.E.: Chloramphenicol resistance in *Campylobacter coli*: nucleotide sequence, expression, and cloning vector construction. Gene 94 (1990a) 23–28.

Wang, Y. and Taylor, D.E.: Natural transformation in *Campylobacter* sp. J. Bacteriol. 172 (1990b) 949–955.

Wassenaur, T.M., Bleumink-Pluym, N.M.C. and Van der Zeijst, B.A.M.: Inactivation of *Campylobacter jejuni* flagellin genes by homologous recombination demonstrates that *flaA* but not *flaB* is required for invasion. EMBO J. 10 (1991) 2055–2061.